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Zebrafish regenerate full thickness optic nerve myelin after demyelination, but this fails with increasing age

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Zebrafish regenerate full thickness optic nerve myelin after demyelination, but this fails with increasing age

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Abstract

Introduction

In the human demyelinating central nervous system (CNS) disease multiple sclerosis, remyelination promotes recovery and limits neurodegeneration, but this is inefficient and always ultimately fails. Furthermore, these regenerated myelin sheaths are thinner and shorter than the original, leaving the underlying axons potentially vulnerable. In rodent models, CNS remyelination is more efficient, so that in young animals (but not old) the number of myelinated axons is efficiently restored to normal, but in both young and old rodents, regenerated myelin sheaths are still short and thin. The reasons for these differences in remyelination efficiency, the thinner remyelinated myelin sheaths compared to developmental myelin and the subsequent effect on the underlying axon are unclear. We studied CNS remyelination in the highly regenerative adult zebrafish (*Danio rerio*), to better understand mechanisms of what we hypothesised would be highly efficient remyelination, and to identify differences to mammalian CNS remyelination, as larval zebrafish are increasingly used for high throughput screens to identify potential drug targets to improve myelination and remyelination.

Results

We developed a novel method to induce a focal demyelinating lesion in adult zebrafish optic nerve with no discernible axonal damage, and describe the cellular changes over time.

Remyelination is indeed efficient in both young and old adult zebrafish optic nerves, and at 4 weeks after demyelination, the number of myelinated axons is restored to normal, but internode lengths are short. However, unlike in rodents or in humans, in young zebrafish these regenerated myelin sheaths were of normal thickness, whereas in aged zebrafish, they were thin, and remained so even 3 months later. This inability to restore normal myelin thickness in remyelination with age was associated with a reduced macrophage/microglial response.

Conclusion

Zebrafish are able to efficiently restore normal thickness myelin around optic nerve axons after demyelination, unlike in mammals. However, this fails with age, when only thin myelin is achieved. This gives us a novel model to try and dissect the mechanism for restoring myelin thickness in CNS remyelination.

Introduction

Regeneration in the human central nervous system (CNS) affected by multiple sclerosis is less efficient than in rodent models of the disease, and the reasons for this are not well understood. Understanding and enhancing the regeneration of myelin sheaths in the CNS (remyelination) are important research aims in multiple sclerosis, where demyelination occurs in the CNS and remyelination ultimately fails. Demyelination of axons reduces nerve conduction velocity by failure of saltatory conduction and causes axonal damage due to loss of metabolic support from the oligodendrocyte to the axon [1,2]. Remyelination does occur in humans [3], but is insufficient, rarely restoring normal numbers of myelinated axons in multiple sclerosis lesions [4,5] and remyelination capacity declines with age [6]. Furthermore, the myelin sheaths produced are thin and short [7], potentially leaving the underlying axon vulnerable [8]. Remyelination in experimental animal models of multiple sclerosis has been shown to restore fast saltatory conduction [9], reduce axonal energy demands [10] and improve functional outcome [11–13]. Hence, enhancing remyelination efficiency, both in terms of extent and quality, may not only restore function after multiple sclerosis relapses but also aid neuroprotection, avoid neurodegeneration and slow or prevent progressive disability. No licenced drugs are yet available for treatment of progressive multiple sclerosis, and both pharmaceutical researchers and academia are interested in understanding how remyelination is regulated to find targets to manipulate its efficiency.

One possible way to discover new targets to enhance remyelination is to study organisms that are more efficient at regeneration. Remyelination in the rodent CNS after toxin mediated demyelinating injury is efficient, so that in young rodents the proportion of axons with a myelin sheath in lesions is restored to that seen in control animals, though this ability reduces with age, associated with impaired oligodendrocyte progenitor cell recruitment and differentiation into myelin forming oligodendrocytes in older animals [14,15]. However, in both young and old rodents, as in humans, regenerated myelin sheaths are also thin and short [16,17].

Zebrafish are remarkable for their regenerative capacity of various organs, including in the CNS [18–20]. They have myelinated axons and are increasingly used to tease apart the mechanisms of myelination and remyelination of the CNS [21–24], and for drug screens looking for compounds altering these processes in larvae [25–27]. However, the efficiency of

remyelination in adult zebrafish in terms of extent or quality is unknown, partly due to lack of a suitable focal demyelination strategy. Here, we use a novel model of focal demyelination of the adult zebrafish optic nerve as a research tool to answer the questions of whether the process of remyelination is similar to mammals, whether these vertebrates with a generally high capacity for regeneration show enhanced remyelination capacity in terms of extent and quality of myelin (compared to rodents and humans), and whether remyelination efficiency declines with age.

Materials and methods

Fish husbandry and housing

All zebrafish lines were kept and raised in our animal facility under standard conditions [28], with a 14 hour light and 10 hour dark cycle at 26.5°C. We used wild type (WIK) and the following transgenic strains: *tg (olig2:DsRed)* [29], *tg (claudink:GFP)* [20], *tg (claudink:GFP/olig2:DsRed)* [20] and *tg (FoxD3:GFP)* [30]. Two ages of zebrafish were used for this study. Zebrafish reach fertility at about 3 months post fertilisation and breed regularly until 12 to 15 months of age. Therefore, to investigate young adults, we used 4 to 7 months old male and female fish of the strains mentioned above. We chose 15 to 18 months to represent an “aged” time point, since natural breeding frequency significantly declines by this age and overall growth of the fish has ceased [31].

Surgical procedures

All experiments were performed under British Home Office regulations.

Optic nerve crush

Optic nerve crush of adult zebrafish was performed as previously described [32,20]. Fish were anaesthetised in aminobenzoic acid ethylmethylester (MS222, Sigma-Aldrich, St Louis, MO, USA; 1:5000 in PBS) and positioned under a stereomicroscope on an ice cold surface. Using fine forceps, the left eye was gently lifted from its socket and the exposed optic nerve was crushed behind the eyeball. A translucent strip across the usually white opaque optic nerve indicated a successful lesion. The eye was rotated back into the socket and the fish placed back into the water. Optic nerve crush was only used as a positive control in the axonal tracing experiments to determine whether LPC caused axonal injury.

Lysophosphatidylcholine (LPC) application to optic nerve

For application of compounds to the optic nerve, small pieces of absorbable gelatin foam (Gelfoam, Pfizer, New York, NY, USA) containing 5 µl of 1% LPC (Sigma-Aldrich, St Louis, MO, USA) in PBS were prepared. Adult fish were anaesthetised as above, placed on an ice cold surface under a stereomicroscope and using fine forceps, the eye was gently lifted out of the socket. A small piece of gelatin foam with LPC was placed next to the optic nerve behind the eyeball, and the eye was placed back into the socket. UK Home Office regulations do not allow bilateral optic nerve operations due to potential blindness and so experimental lesions were either compared to the unlesioned contralateral optic nerve, or to PBS on gelatin foam applied to the optic nerve of another fish.

Axonal tracing

The optic nerve was exposed as described above. The tracer was applied to severed axons of the optic nerve using small pieces of biocytin-soaked gelatin foam and the tracer was allowed to be anterogradely transported along the optical projection for 4 hours [32].

Tissue processing and immunohistochemistry

Fish were terminally anaesthetised in MS222 (1:1000 in PBS), perfusion fixed with 4% paraformaldehyde (PFA) and the dissected tissue was either a) embedded in 4% agar in PBS and cut into 50 μ m sections on a vibratome or b) immersed in 30% sucrose in PBS overnight, embedded in cryostat embedding medium, frozen and cut into 14 μ m sections on a cryostat or c) dissected optic nerve was post-fixed in 4% PFA for 15 min, teased into single fibres on superfrost glass slides and dried. Colorimetric staining was performed with the Vectastain ABC kit (Vector Laboratories Ltd). Immunofluorescent staining was performed as described previously [20].

Antibodies and specificities: rat anti-Claudin k (1:1000, developed by us and specific for zebrafish Claudin-K [20]). Mouse anti-4C4 (1:50, Developmental Studies Hybridoma Bank (DSHB), 7.4.C4 mouse hybridoma cell line, developed to an unknown teleost antigen and previously characterised to recognize microglial cells by morphological criteria after optic nerve lesion [33] and spinal lesion [34] transforming from stellate cells into amoeboid cells, increasing in number along lesioned tracts and containing fluorescently labelled cellular debris indicating phagocytic activity.) Mouse anti-LINC (1:5, DSHB, antibody recognizes axonal filaments of 56 and 58 kD in amphibians [35] and its specificity for long projecting axons, especially optic axons, has previously been demonstrated by immunohistochemistry in zebrafish [36]. Mouse anti-pan-sodium channel antibody (1:200, Sigma-Aldrich monoclonal antibody from Clone K58/35, raised to sequence conserved between all vertebrates and shown to be specific to sodium channels in zebrafish by co-localisation with other nodal proteins and complementary expression to internodal markers [37]).

Electron microscopy

Adult zebrafish were perfused with primary fixative (2% glutaraldehyde/ 4% formaldehyde in 0.1 M sodium cacodylate in PBS, pH 7.4) and post-fixed at 4°C overnight. Samples were stimulated in a microwave in secondary fixative (2% osmium tetroxide in 0.1 M cacodylate and 0.1 M imidazole in PBS, pH 7.5), (100 watts for 1 min, room temperature for 1 min, 100 watts for 1 min, then 450 watts for 20 sec/room temperature for 20 sec repeated 5 times), then rinsed and stained with saturated (8%) uranyl acetate. To dehydrate the samples, they were microwave stimulated (250 W) in each of a series of increasing concentrations of ethanol to 100% acetone. Samples were put in a 1:1 mix of EPON embedding medium (Embed 812 kit, Electron microscope sciences) and acetone overnight, placed in pure EPON for >6 hours, embedded and incubated at 60-65°C to polymerise for > 24 hours, before sectioning. Ultra-thin sectioning and lead staining was performed by Steven Mitchell in the Science Faculty Electron Microscope Facility, University of Edinburgh.

Quantification

All quantification was carried out blinded to treatment group.

- a) *Pixel intensity and cell counts*: quantified in a defined area of interest (0.01 mm^2) within the lesion site (located 300–400 μm from the optic chiasm) and an equally sized and located area in the contralateral, unlesioned optic nerve.
- b) *Axonal tracer experiments*: the pixel darkness of the entire optic tectum in six randomly chosen cross sections was compared to the unlesioned contralateral side.
- c) *G-ratios, axon diameters and the percentage of myelinated axons*: the lesion area was identified in serial cross-sections by distance from the chiasm, presence of myelin debris and/or macrophages with engulfed myelin droplets. To ensure a random selection of axons to be quantified, without bias to presence or absence of myelin or shape, grids were overlaid onto the images and all axons intersecting with the grid lines [38] were counted as myelinated or not (percentage of myelinated axons), and measured for axon and myelin thickness (G-ratio) by a blinded observer. To obtain the axon diameter, the circumference of the axon was measured with a Bamboo Pen and Touch pad (Wacom, Vancouver, WA, USA), and the diameter was calculated with the formula: $\text{diameter} = (\text{circumference}/\pi)$. Myelin thickness was measured similarly in the same axons, by tracing the circumference of the myelinated axon, calculating the diameter of the fibre and subtracting axon diameter from fibre diameter. To examine the range of axon diameters seen, frequency-distribution plots were generated by counting the number of axons present in each axon diameter range bin. This is presented as a percentage frequency graph, cumulative frequency graph and a box and whisker plot showing median value, with interquartile ranges, with maximum and minimum points as the ends of the whiskers. This is to allow comparison of data which is not normally distributed. For all of these measurements, at least 200 axons were measured per animal, and at least 3 animals were analysed per condition ($n \geq 3$).
- d) *Internode lengths*: in teased fibre preparations of the optic nerve, 80–100 internodes from each of $n = 3$ zebrafish were measured and the frequency distribution graph analysed by the Kolmogorov-Smirnov test.

Statistics

The statistical tests used are specified in the text and figure legends. Experiments with two variables were analysed by the Mann Whitney U-test and experiments with three or more variables by an ANOVA and Tukey's post test, (if normal distribution of data) or Kruskal Wallis test and Dunn's post test, (if data was not normally distributed). A p value of 0.05 or less was considered statistically significant (* denotes $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Results

Lysophosphatidylcholine (LPC) treatment of the adult zebrafish optic nerve leads to a focal demyelinating lesion followed by remyelination within 4 weeks

Although crush lesions of the optic nerve of zebrafish (and the closely related goldfish) to study re-establishment of myelin on regenerated axons have been carried out previously [39,40], these studies do not assess remyelination, because myelination of a newly generated axonal sprout may show differences to remyelination of a demyelinated axon. To study remyelination, we aimed to produce a model of focal demyelination, with no or minimal axonal damage. The optic nerve is ideal for remyelination studies as it contains mostly

myelinated axons, is easily accessible and has been used previously for investigation of axon regeneration [41,20]. We used the myelinotoxin LPC, as it has been used extensively in rodent models of demyelination (reviewed in [16]). This was soaked onto a small piece of absorbent gelatin foam placed next to the adult zebrafish optic nerve to produce focal demyelination; the contralateral optic nerve served as an internal control. Application of gelatin foam containing PBS only to the optic nerve led to no demyelination, axonal damage or cellular disturbance (Additional file 1: Figure S1).

In rodents, after injection of myelinotoxin (LPC or Ethidium Bromide), there is maximal demyelination at day 3, oligodendrocyte precursor cell (OPC) recruitment begins around day 6, OPC maturation into oligodendrocytes starts around day 10 and remyelination is complete by day 28 [42,43]. Microglia/macrophages are seen within 6–12 hours until 4 days after LPC-induced demyelination [44]. For an overview of the timeline of demyelination and remyelination in zebrafish optic nerve, we quantified the intensity of immunoreactivity for the myelin marker Claudin k [20], the number of oligodendroglial cells (olig2-positive cells from reporter transgenic fish), microglia/macrophages (cells positive with anti-4C4 antibody [34]) and axons (intensity of anti-LINC antibody immunofluorescence [35,20] at different time points after the lesion (Figure 1). We used the Olig2-reporter fish as a tool to detect oligodendroglial cells (98% of Olig2+ cells are oligodendroglial in the adult zebrafish [45]), due to the lack of an antibody marker to reliably detect oligodendroglia of any stage in the lineage in fish. This reporter labels predominantly OPCs [46] but also more mature oligodendroglial cells [20,45]. By combining this reporter fluorescence with an immunofluorescence antibody stain of a myelin marker such as Claudin k, we were able to study the dynamics of oligodendroglial cells (mostly OPCs) and myelin formation separately. Although myelin markers also label mature oligodendrocyte cell bodies, it is impossible to see these clearly in heavily myelinated tracts of adults. Claudin k immunofluorescence in the lesioned optic nerve was unchanged at 4 days post lesion (dpl) compared to the contralateral control side, but reduced at 8 dpl ($***p < 0.001$, ANOVA and Tukey's post test) and this gradually increased so that by 28 dpl, immunofluorescence labelling had recovered to similar values as in the contralateral control side (Figure 1A). The number of olig2-positive oligodendroglial cells in the lesion area was already significantly reduced at 4 dpl, remaining low at 8 dpl ($*p < 0.05$, $***p < 0.001$ respectively, ANOVA and Tukey's post test) and gradually returning to levels comparable to the contralateral control optic nerve by 21 dpl (Figure 1B). The number of 4C4-positive microglia/macrophages was increased in the demyelinated optic nerve at 4 dpl ($p < 0.001$, ANOVA and Tukey's post test) (Figure 1C) and returned to control levels by 14 dpl. There was no change in LINC immunoreactivity over this time course suggesting a lack of axonal injury (Figure 1D). At least five fish ($n \geq 5$) were used for each time point. Thus our model results in focal demyelination, characterised by reduced oligodendrocyte precursors, decreased myelin and a microglial/macrophage response, with remyelination occurring within 28 dpl.

Figure 1 Characterisation of myelin, oligodendroglia, microglia/macrophages and axons following demyelinating lesion produced by LPC application in the adult zebrafish optic nerve. (A-D) There is (A) a significant reduction in Claudin k (myelin) immunoreactivity (arrows, $p < 0.001$), (B) fewer olig2:dsred positive oligodendroglia in the lesion site at 4 to 14dpl (arrows, $p < 0.05$), (C) transiently increased numbers of 4C4 immunoreactive microglia/macrophages at 4 to 8dpl (arrows, $p < 0.001$) and (D) no change in LINC + axon pixel intensity. Pixel intensities in A and D are presented as a ratio between lesioned nerve and unlesioned contralateral control. Dashed lines outline optic nerve. A minimum of $n = 5$ fish were used for each time-point. Mean \pm SEM. All ANOVA and Tukey's post test. Scale bar: A-D = 100 μ m.

Electron microscopy and axonal tracing indicate intact axons after LPC treatment

The lack of change in LINC immunoreactivity over time suggests that most axons are not damaged by the LPC treatment, however, this may be masked by sprouting axons, normally seen after axonal damage [40,41]. Using electron microscopy, at the time of maximal demyelination (8 dpl), we compared cross sections of optic nerve taken through focal demyelinated lesions with those from a crushed optic nerve to look for signs of axonal sprouting. After nerve crush, we saw many small calibre axons, arranged in clusters and indicative of axonal sprouting secondary to damage, but after LPC treatment, there were only unmyelinated average-calibre axons (Figure 2A). Frequency distribution analysis of axon diameter after crush, demyelination and in control (uncrushed) optic nerve indicates increased numbers of small calibre axons following optic nerve crush only, suggesting that LPC-treatment does not induce nerve sprouts (Figure 2B). Furthermore, although the median axon diameter is virtually identical in control and LPC-treated nerves, there is also a higher frequency of larger axons in LPC-treated nerve compared to control nerve. This was unexpected but not unprecedented as an increase in axon diameter after demyelination has been previously described in MS *post mortem* tissue, postulated related to increased axon water content [47] and in damaged rat optic nerve that shows uncompacted myelin [48].

Figure 2 Electron microscopic analysis and axonal tracing shows no axonal damage after LPC induced demyelinating lesions. (A) In a demyelinated optic nerve (left), unmyelinated axons of various axonal diameters are seen compared to a crushed optic nerve (right) where axonal diameters are smaller. **(B)** To quantify axon diameter, 200 axons of each of $n = 3$ fish were measured per condition and are shown as frequency of axons per axon diameter bin. This is also shown as a cumulative frequency, and a box and whisker plot (showing median, interquartile range and maximum and minimum values). These representations all illustrate that small diameter axons are more frequent after nerve crush than in unlesioned controls or after LPC treatment. Furthermore, large diameter axons are more frequent after LPC treatment. **(C)** Tracer was applied to optic nerve (cut proximal to lesion) 8 days after treatment with LPC (or PBS for control) so that with no axon damage, the tracer labels the entire contralateral optic tectum (top picture), but with axon damage, partial tectal labelling is observed (bottom picture). **(D)** There are no differences in tracer labelling in brains after treatment with LPC or PBS-control, whereas a partial crush results in distinct areas of lower labelling intensity (black arrows), (dorsal is up), also seen by quantification of tectal labelling compared to the contralateral unlabelled control side **(E)** ($p < 0.05$, $n = 3$ fish, Kruskal Wallis test, Dunn's post-test). Mean \pm SEM. Scale bars: A = 1 μ m, D = 200 μ m (top), 100 μ m (bottom).

To test whether functional axonal integrity after LPC-induced demyelination is retained in this model, we used axonal tracing. Zebrafish optic nerves cross at the optic chiasm and, unlike in mammals, all axons from the right eye cross to the left optic tectum and vice versa [49]. We applied an axonal tracer to cut optic nerve proximal to a focal demyelinated lesion, partial optic nerve crush and sham treated control at 8 dpl, and quantified tracer anterogradely transported to the contralateral optic tectum (Figure 2C). If axons are damaged, tracer will not reach the tectum leading to absent staining, and in the event of no damage, tracer will be transported to the entire tectum and result in visible staining. Our results show that there was no difference between the pattern or quantification of tracer labelling in the optic tectum in control treated nerve and focally demyelinated nerve, but there was a reduction of tracer found in the optic tectum after partial optic nerve crush with obvious areas of lower intensity labelling ($p < 0.05$, $n = 3$ fish, Kruskal Wallis test, Dunn's post test, Figure 2D, E). Unchanged LINC immunoreactivity, lack of evidence for sprouting by electron microscopy and unimpeded tracer transport gave us confidence that our focal demyelination model produces no detectable axonal damage allowing us to investigate true remyelination.

In young zebrafish, optic nerve axons are remyelinated completely with full thickness myelin

To evaluate remyelination, we examined both the number of axons that were remyelinated at each time point and the thickness of the myelin sheath in young adult zebrafish (4–7 months). Cross sections through the lesion area examined by electron microscopy showed that at 8 dpl, most axons were demyelinated compared to the unlesioned contralateral control optic nerve ($24\% \pm 9\%$ vs. $93\% \pm 1\%$ myelinated axons, mean \pm SEM, $p < 0.05$, $n = 3$ fish, Kruskal Wallis test, Dunn's post-test). At 28 dpl, the percentage of myelinated axons was the same as in the unlesioned contralateral control optic nerve ($92\% \pm 1\%$, vs $93\% \pm 1\%$ myelinated axons, mean \pm SEM) (Figure 3A, B), in agreement with the pattern of Claudin k immunoreactivity (Figure 1).

Figure 3 In young zebrafish the proportion of myelinated axons and myelin thickness is fully restored after demyelination. (A) In lesioned optic nerve, electron microscopic analysis shows many demyelinated axons at 8 dpl (asterisks) and complete remyelination at 28 dpl compared to unlesioned controls. (B) At 8 dpl the number of myelinated axons in the lesion area is significantly reduced compared to unlesioned controls ($p < 0.05$, $n = 3$ fish, Kruskal Wallis test, Dunn's post-test), while there is no difference between remyelinated and control optic nerves. (C) Graphs of G-ratio versus axon diameter show similar patterns at 28 dpl and in unlesioned controls, while most axons at 8 dpl have a higher G-ratio (less myelin). (D) Average G-ratio between unlesioned and remyelinated optic nerve is not different, indicating remyelination with normal thickness myelin ($p > 0.05$, $n = 3-6$ fish, Mann Whitney U-test). Mean \pm SEM. Scale bar: A = 1 μ m.

A typical finding of remyelination in humans [3] and rodents [50] is a thinner myelin sheath than expected for axon diameter, measured using the G-ratio: ratio of axon diameter to axon plus myelin sheath diameter. To determine whether this is similar in zebrafish, we measured the G-ratio in cross sections through the lesion site in remyelinated optic nerves, compared to the unlesioned control side. Scatter graphs of G-ratio versus axon diameter at 8 dpl show high G-ratios approaching one, indicative of demyelination. At 28 dpl, G-ratios are indistinguishable from unlesioned controls, showing full myelin thickness and compact myelin sheaths (Figure 3A, C, D). In mammals, Schwann cells, which myelinate axons of the peripheral nervous system (PNS), produce myelin with lower G-ratios (thicker myelin)

compared to myelin produced by oligodendrocytes [51], and are known to be able to remyelinate the mammalian CNS after injury [52,53]. To determine whether Schwann cell remyelination accounted for the full thickness myelin observed in remyelinated zebrafish optic nerve, we made use of the *tg (FoxD3:GFP)* transgenic zebrafish line, which labels neural crest-derived Schwann cells [30]. This showed that at 28 dpl, no GFP-positive cells were present in the remyelinated optic nerve, however GFP-labelled cells in peripheral nerves could be seen in muscle fascicles close by (Figure 4A) confirming that the transgene was still active in the adult zebrafish. In addition, electron microscopy analysis of remyelinated optic nerve cross sections did not show any visible basal laminae (only seen around Schwann cells), nor Schwann cell-like myelin profiles (Figure 4B), distinguishable from oligodendrocyte myelin profiles by the close association of Schwann cell nucleus and myelinated axon (one-to-one relationship) (Figure 4C). Hence, neural crest-derived Schwann cells do not account for the normal G-ratios seen here. After demyelination, young adult zebrafish do not only regenerate myelin sheaths around all axons, but also quickly restore the full thickness of myelin – different from both rodents and humans.

Figure 4 Schwann cells are not involved in optic nerve remyelination. (A) There are no GFP positive cells in the remyelinated optic nerve of FoxD3:GFP transgenic zebrafish (labelling neural crest-derived Schwann cells) at 28 dpl whereas peripheral nerves in nearby muscle fascicles are detectable (arrows), confirming expression of the transgene. (B) Schwann cells are also not detected in electron microscopic analysis of remyelinated optic nerve cross sections. (C) Schwann cells have a typical phenotype: the nucleus is closely associated with its myelinated axon, with a basal lamina. Scale bars: A = 100 μ m, B = 1 μ m.

Remyelination is impaired in old zebrafish

Regeneration potential decreases with age in many mammalian tissues, including the CNS [54], and zebrafish are also known to have altered neural function with increasing age [55]. We therefore tested whether aged zebrafish are still capable of such efficient myelin repair. We induced focal demyelinating lesions in old zebrafish (>15 months) and determined the number of remyelinated axons and myelin thickness as before. We found that following optic nerve demyelination, the percentage of axons that were remyelinated at 28 dpl in old zebrafish was no different from that in fully remyelinated young zebrafish or in contralateral control optic nerves from the same animals (Figure 5A, B). However, the myelin sheaths in old zebrafish at 28 dpl were noticeably thin (higher G-ratio), and these remained thin even at 3 months post lesion (mpl) ($p < 0.05$, 3 animals per condition and 150–200 axons per animal, Kruskal-Wallis test, Dunn's post test, Figure 5A, C, D). Therefore, the thin myelin observed at 28 dpl is not simply a result of slower and incomplete remyelination in old zebrafish which is correctable in time. Therefore, old zebrafish retain the ability to remyelinate all axons, but lose the ability to restore this myelin to full thickness.

Figure 5 Remyelination is impaired in old zebrafish. (A) Electron microscopic analysis of the lesion area in the optic nerves of old (>15 months) zebrafish show thin myelin at 28 dpl and even 3 mpl. (B) The percentage of myelinated axons in old fish recovers to a similar level as in controls and young fish. ($p > 0.05$, $n = 3$ fish, Kruskal-Wallis test, Dunn's post test). (C) G-ratios in old zebrafish at 28 dpl and 3 mpl are higher than in unlesioned controls, indicating thin newly formed myelin following demyelination, confirmed by (D) quantification of average G-ratios at different time points post-lesion ($p < 0.05$, $n = 3$ fish, Kruskal-Wallis test, Dunn's post test). Data from young animals in B and D are repeated from Figure 3 for comparison. Mean \pm SEM. Scale bar: A = 1 μ m

Internodal lengths are shorter after remyelination in young and old zebrafish

Another typical feature of remyelination in mammals is that the internodes are short [50,56]. We measured internodal lengths in teased optic nerve fibres of young and old zebrafish after remyelination, compared to the non-lesioned side (Figure 6). Internode length in adult control optic nerve fibres did not change with age. However, after remyelination, the average internodal length was decreased in both young and old zebrafish compared to the unlesioned control optic nerves from the same animals (Young lesion: $17.48 \pm 3.22 \mu\text{m}$; young control: $23.51 \pm 0.59 \mu\text{m}$; old lesioned: $15.31 \pm 1.12 \mu\text{m}$; old control: $22.32 \pm 3.51 \mu\text{m}$; $n = 3$, all mean \pm SEM). A frequency-distribution histogram shows a higher frequency of short internodes (1–20 μm) in lesioned teased fibres of young and old zebrafish than unlesioned control optic nerves of the same animals (Figure 6A-C) ($p < 0.001$, 80–100 axons in each $n = 3$ animals, Kolmogorov-Smirnov test). Thus, after remyelination, as in mammals, myelin internodes are shorter in zebrafish, regardless of age.

Figure 6 Internodes on remyelinated optic axons are short in young and old zebrafish.

(A, B) Frequency distributions of internodal lengths after remyelination in young (28 dpl) and old (3 mpl) zebrafish are different from those in age-matched unlesioned controls ($p < 0.001$, Kolmogorov-Smirnov test, 80–100 internodes measured per animal in $n = 3$ animals). Shorter internodes (1–20 μm) are more frequent in remyelinated young and old zebrafish than in controls. (C) Teased-fibre preparations illustrate shorter internodal distances on remyelinated optic axons. Arrows - nodes stained with a pan-sodium channel marker. Mean \pm SEM. Scale bar: C = 6 μm .

Old zebrafish show a reduced microglial/macrophage response after demyelination

What causes this inability to regenerate full thickness myelin in old zebrafish? One of the mechanisms suggested to underlie failure of remyelination in old mice is decreased recruitment of microglia/macrophages to the lesion site [57], thought important in myelin debris clearance and cytokine signalling. For this reason, we investigated whether a reduced microglial/macrophage response to demyelination occurred in old zebrafish. We found fewer 4C4-positive microglial/macrophages 4 days post lesion in old zebrafish compared to young zebrafish ($p < 0.05$, $n = 5$ fish, Kruskal Wallis test, Dunn's post test, Figure 7A, B), with no difference in the number of microglia/macrophages compared to control optic nerve. Although this is only associative, insufficient recruitment of microglia/macrophages to the lesion site at this early time point may contribute to an inability to restore full thickness myelin through remyelination in old zebrafish.

Figure 7 The microglial/macrophage response is attenuated in old zebrafish. (A) The number of microglia/macrophages in the lesion area in old zebrafish is similar to that of age-matched unlesioned controls and significantly different from young zebrafish at 4 dpl ($p < 0.05$, $n = 5$ -6 fish, Kruskal Wallis test, Dunn's post test). Representative images of old zebrafish are shown in (B); for images of young zebrafish, please refer to Figure 1C. Dashed lines outline optic nerve. Mean \pm SEM. Scale bar: B = 100 μm .

Discussion

In this study, we use a model of focal demyelination in the adult zebrafish optic nerve, without detectable axonal damage, to study remyelination and find that the zebrafish not only fully regenerates the normal proportion of myelinated axons in a demyelinated optic nerve lesion, but also restores full myelin thickness – a capacity that it loses with age and which is absent in rodents and humans.

Although LPC-induced focal demyelination has been used extensively in rodents (reviewed in [16]) and even goldfish [39,40], it has not previously been described and characterised in adult zebrafish. We found that there is a similar time course and cellular response to that found in mammals following focal LPC-induced lesions [58]. There is restoration of the normal percentage of myelinated axons in lesions at 28 dpl, however in contrast to rodents, macaques [59] and humans, young zebrafish are able to remyelinate with full thickness myelin (as measured by electron microscopy and with no evidence of Schwann cell involvement). The myelin of remyelinated axons of mammals has consistently been shown to be thinner both in remyelinated lesions of multiple sclerosis patients [3,7] and in rodent models of demyelination/remyelination [50], and along with shorter internodes, thin myelin is used as the gold standard for detection of remyelination. It was recently reported that in a traumatic spinal lesion model in mouse, newly formed myelin sheaths were of full thickness [60], but spinal cord injury also causes axonal degeneration, and the control of myelination of regenerated versus demyelinated axons may differ. Even in the PNS of mammals, where remyelination is more efficient, remyelinated axons have thin myelin [61]. In the PNS, myelin thickness is determined by amount of axonal expression of Neuregulin [62], but the mechanism is not fully understood in the CNS, and several pathways have been implicated including IGF-1 [63], Akt [64], PTEN [65] and pERK1/2 [66].

Although, in young zebrafish, oligodendrocytes quickly remyelinate the optic nerve with full thickness myelin, albeit with shorter internodal lengths, old zebrafish do not. In old zebrafish, the same proportion of axons was remyelinated in the same time frame as in young animals, but with thin myelin sheaths. This is unlikely to reflect an age-related slowing of remyelination, as myelin sheaths remain thin even 3 months after the lesion, but instead an age-dependent decline in the quality of remyelination. There is a reduction in the number of remyelinated axons with age both in humans [6] and in rodents [14] but remyelinated myelin sheaths always are thin.

Whether thin and short myelin has detrimental functional consequences has been a key question since the 1940s. Theoretical studies predict that at a fixed axon diameter, increasing the myelin thickness [67] or the internodal length [68] leads to an increase in conduction velocity, until this reaches a plateau. The latter has recently been confirmed *in vivo* in mouse where young transgenic mice engineered to have short internodes have reduced nerve conduction, which normalises during postnatal growth when internodal lengths increase [69]. Thus the reduced internode length after remyelination may simply be related to a lack of growth of the animal in adulthood compared to development. A lack of growth of axon diameter in adulthood has also been postulated as a reason for the thin myelin usually associated with remyelination compared to development, allowing for deposition of only a standard amount of myelin (as is possible around glass nanofibres [70]) but which cannot be modulated to the ideal thickness for the axon diameter [71]. However, we do not see a change in axon diameter between young and old fish, suggesting other mechanisms may be more important. Axon signals modulate the myelinating potential of individual oligodendrocytes at

least in zebrafish development [21], but whether the mechanism is the same in regeneration is currently unknown. Our observation that young zebrafish have normal thickness of myelin but still short internodes after remyelination leads us to suggest that myelin thickness and internodal length are controlled by different mechanisms. Thin myelin and short internodes may increase the vulnerability of remyelinated axons to neurodegeneration in the longer term [72,8] which is relevant in multiple sclerosis pathology to avoid progressive disability accumulation.

Although the mechanism for thin myelin and short internodes in CNS remyelination is not understood in any animal, the reduction of efficiency in the extent of remyelination in aged rodents may be secondary to both a delay of OPC recruitment and slower differentiation into oligodendrocytes [15] as well as an altered microglial/macrophage phenotype and failure of recruitment, leading to reduced clearance of debris and differences in secreted pro-regenerative factors [57,73]. It is not known if these differences affect myelin thickness as well as its extent during remyelination. In our model, old zebrafish exhibit an early blunted microglial/macrophage response compared to young fish, suggesting that the innate immune response may be important, but currently we do not have the tools to differentiate between different microglial/macrophage activation states in zebrafish.

Conclusions

The reason why zebrafish are better at remyelination (and other forms of regeneration) than other species is unknown. Macaques show almost no remyelination of the optic nerve three months after LPC injection, despite successful and fast remyelination of the spinal cord in a similar operation [59], and human multiple sclerosis patients often do not have full recovery from optic neuritis. Our model of focal demyelination and remyelination in the adult zebrafish shows both similarities and differences to mammalian remyelination. The similarities of cells involved and time course of remyelination (compared to mammals) suggest that high throughput screens of small molecule libraries in larval zebrafish may indeed identify potential targets for aiding remyelination. The differences may also prove to be useful, as although zebrafish are highly regenerative compared to humans, this potential limitation may prove an advantage to understand how myelin thickness is successfully regenerated. Our model is an intentionally reductionist model of pure demyelination and remyelination, lacking the complexities of adaptive immune response changes found in multiple sclerosis patients and is clearly not high throughput and not suited to screening for pro-remyelinating targets. However, it may be useful for the further testing of candidate pro-remyelination compounds that may be translatable into neuroprotective therapeutics for multiple sclerosis in the future [74]. It may be even more instructive to answer the fundamental questions of how the extent and quality of repaired myelin is controlled, how ageing affects these processes and what differences in remyelination biology between zebrafish and mammals determine success or failure of regeneration.

Abbreviations

dpl, Days post lesion; LPC, Lysophosphatidylcholine; mpl, Months post lesion; OPC, Oligodendrocyte precursor cell; PBStx, Phosphate Buffered Saline with TritonX-100; PFA, Paraformaldehyde; CNS, Central nervous system; PNS, Peripheral nervous system; SEM, Standard error of means; GFP, Green fluorescent protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EJM carried out the experiments, participated in their design under the supervision of TB and AW, CGB advised and participated in experimental design, TB and AW contributed equally to the conception of the study, participated in its design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

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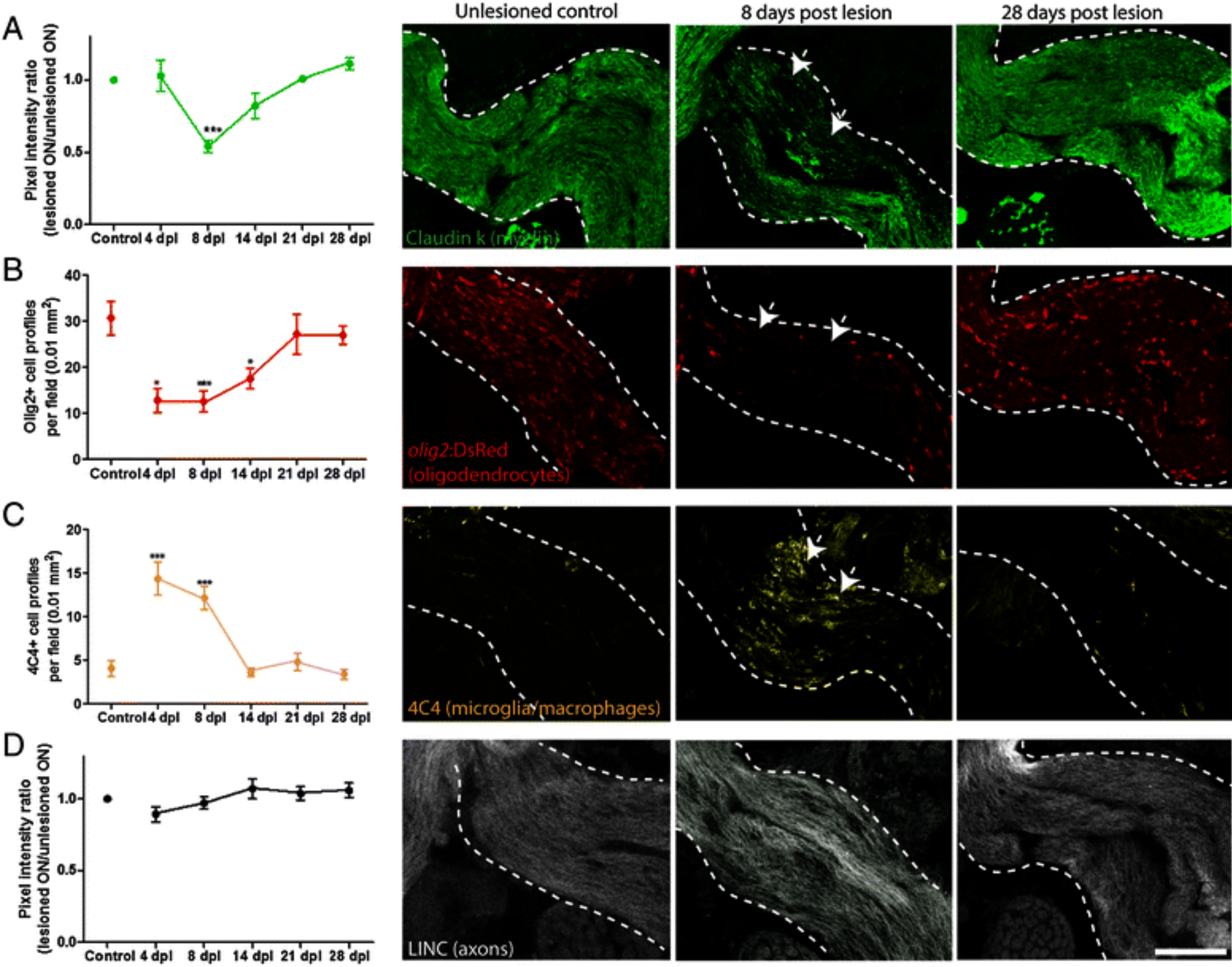
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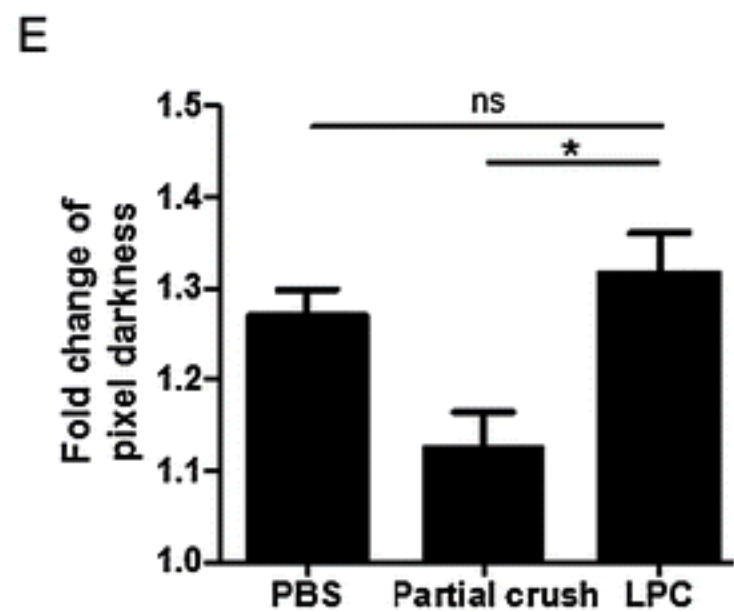
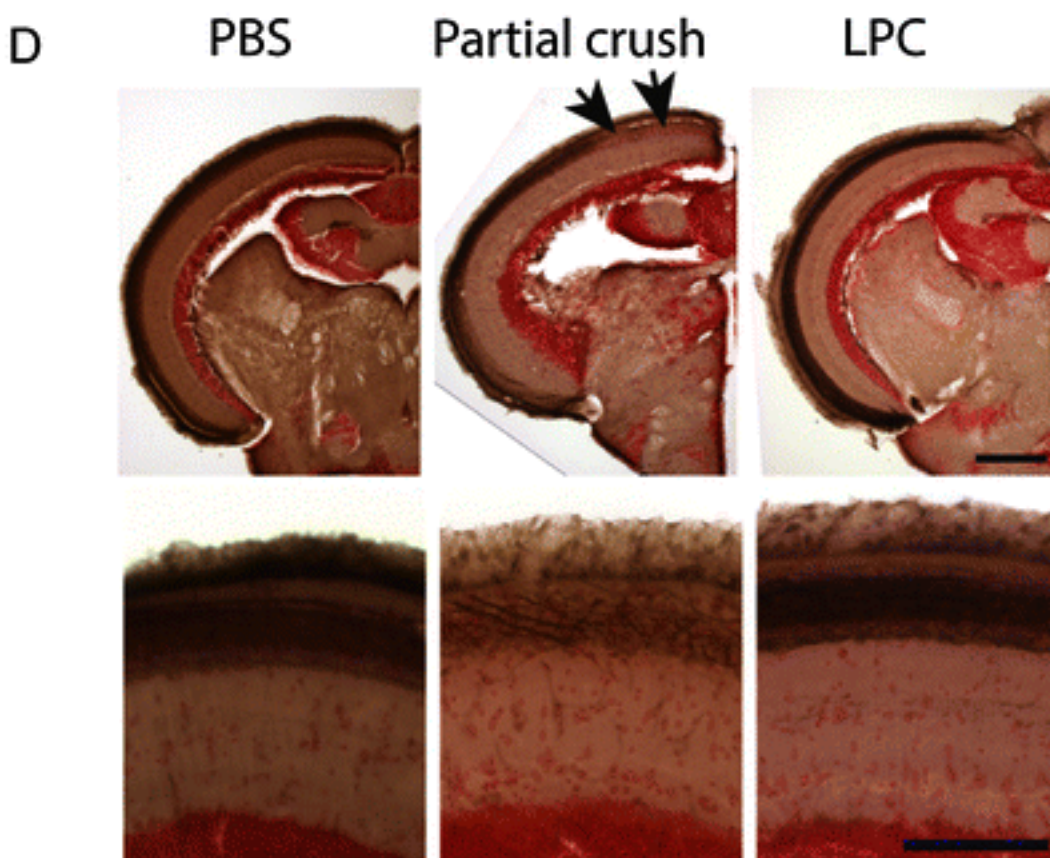
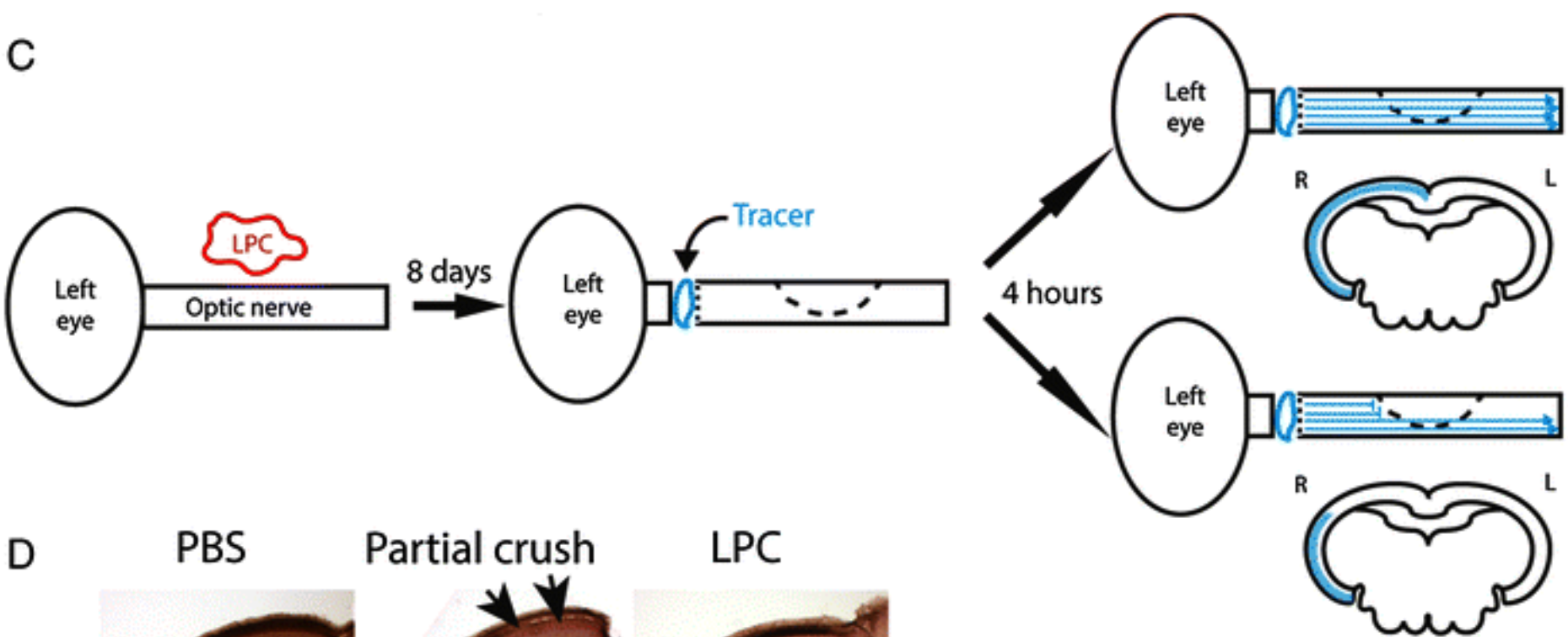
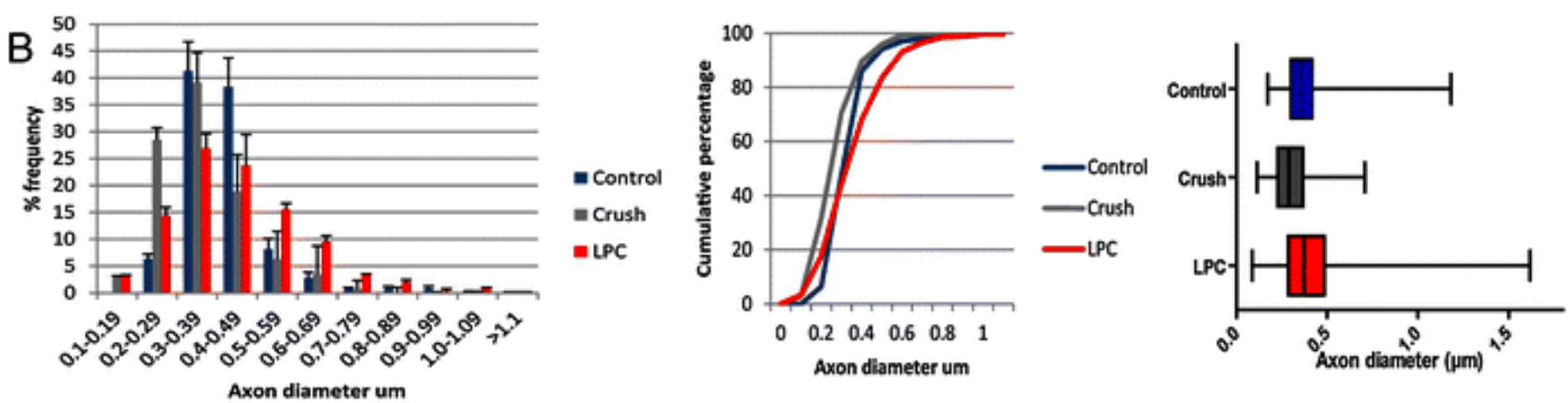
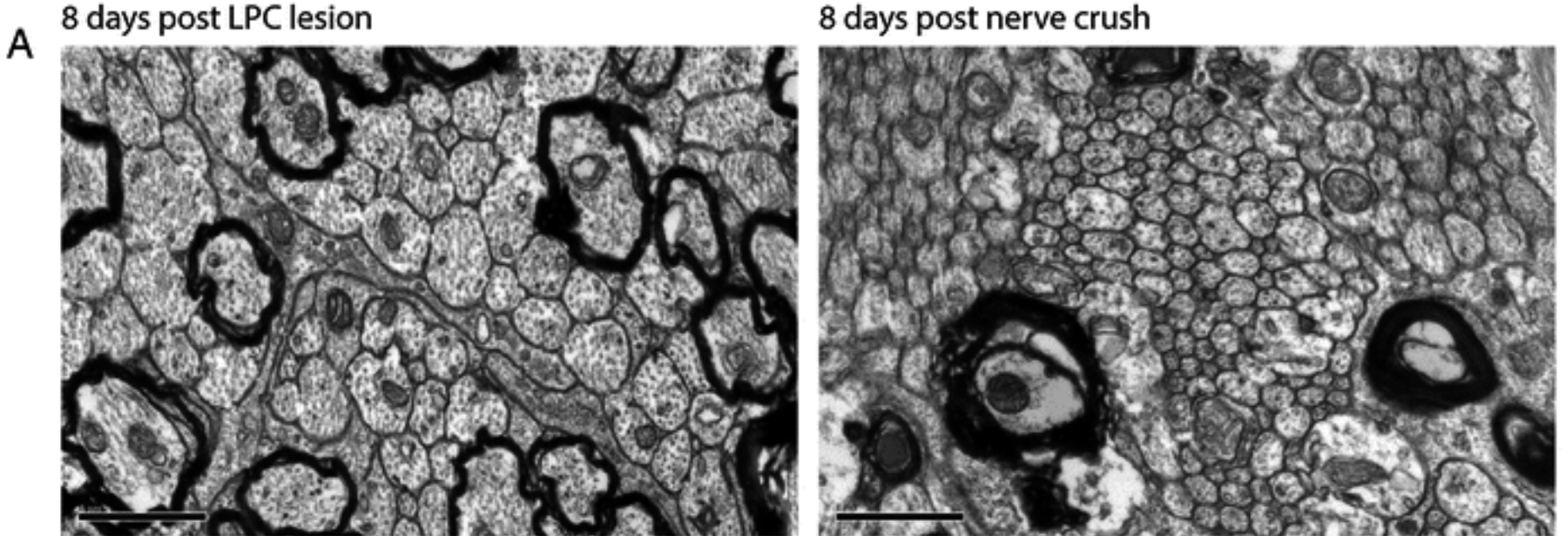
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Additional file

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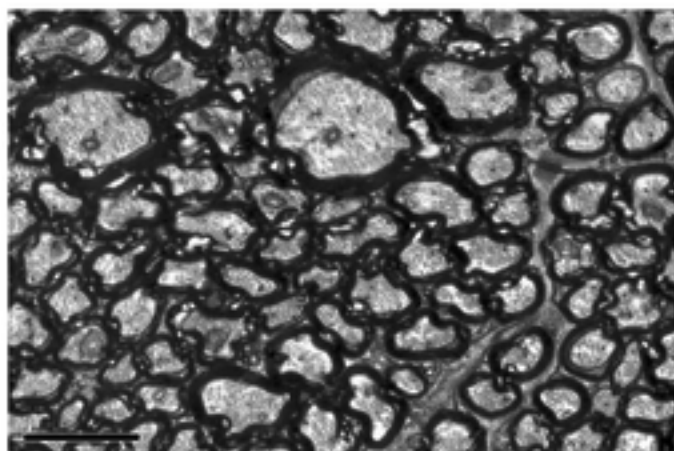
Additional file 1: Figure S1 PBS on gelatin foam alone does not cause demyelination, axonal damage or cellular disturbance. PBS on gelatin foam applied to zebrafish optic nerve does not cause demyelination as measured by (A) lesion size or (B) myelin levels (Claudin k), nor a change in axon staining intensity (B), microglia or oligodendrocytes (C) as compared to a negative unlesioned control side (also see Figure 1). (Mean+/- SEM Kruskal Wallis test, plus Dunn's post-test * $p < 0.05$, ** $p < 0.01$, ns = not significant).



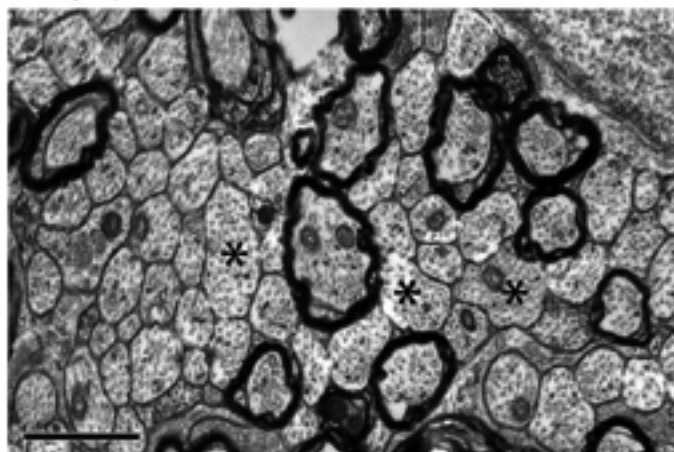


A

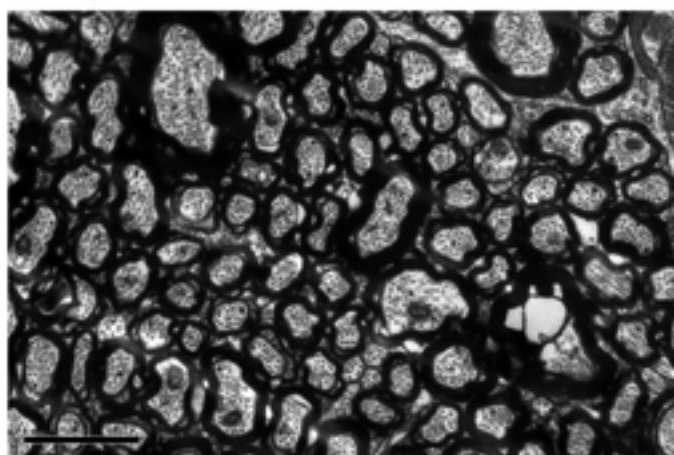
Unlesioned Control



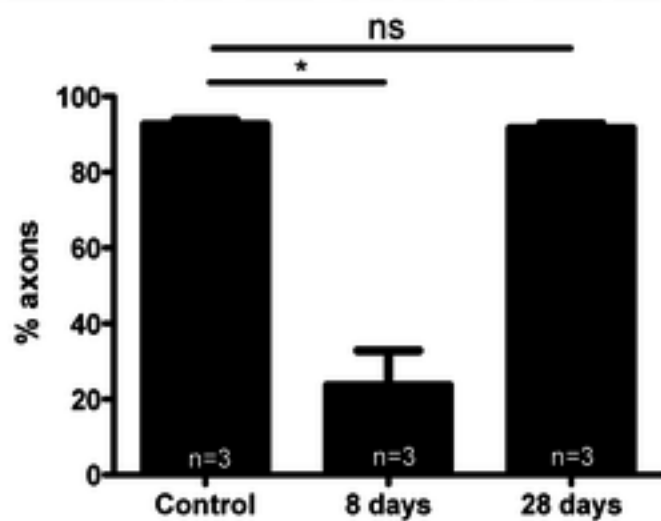
8 days post LPC lesion



28 days post LPC lesion

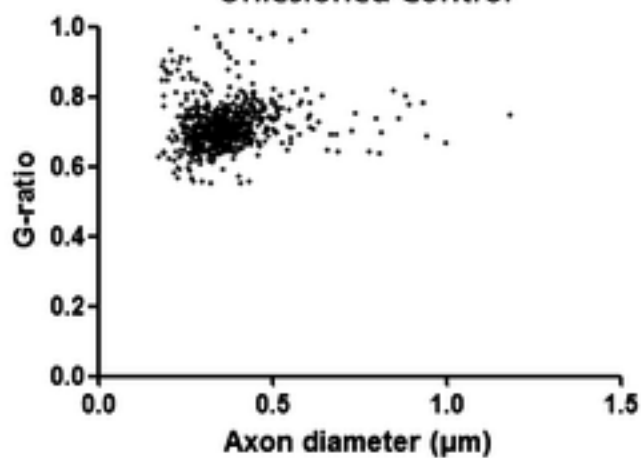


B

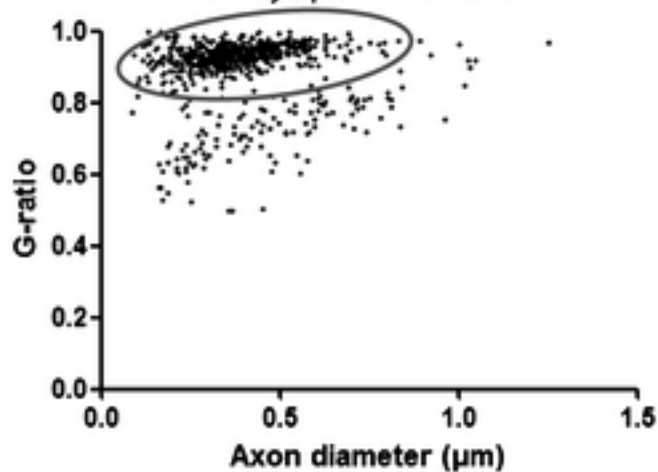


C

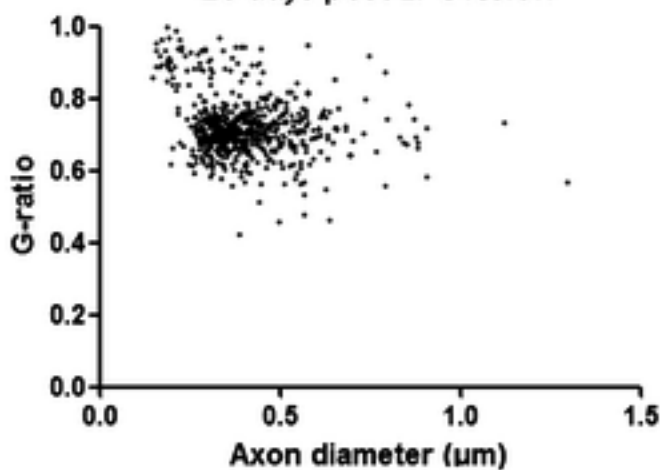
Unlesioned Control



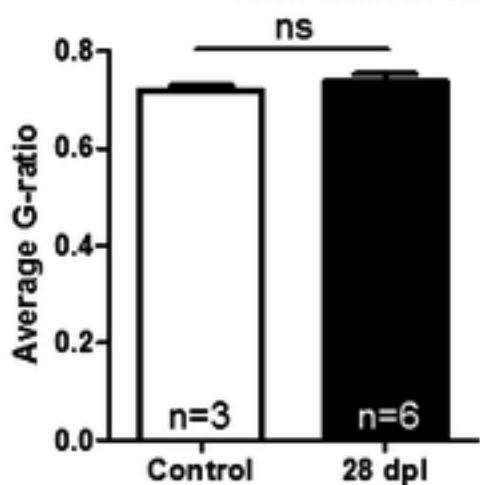
8 days post LPC lesion



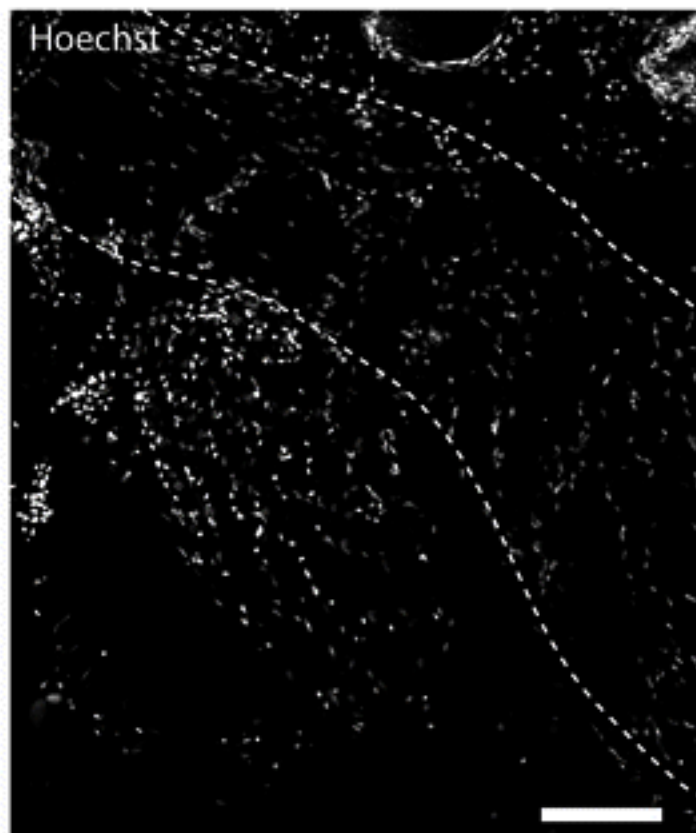
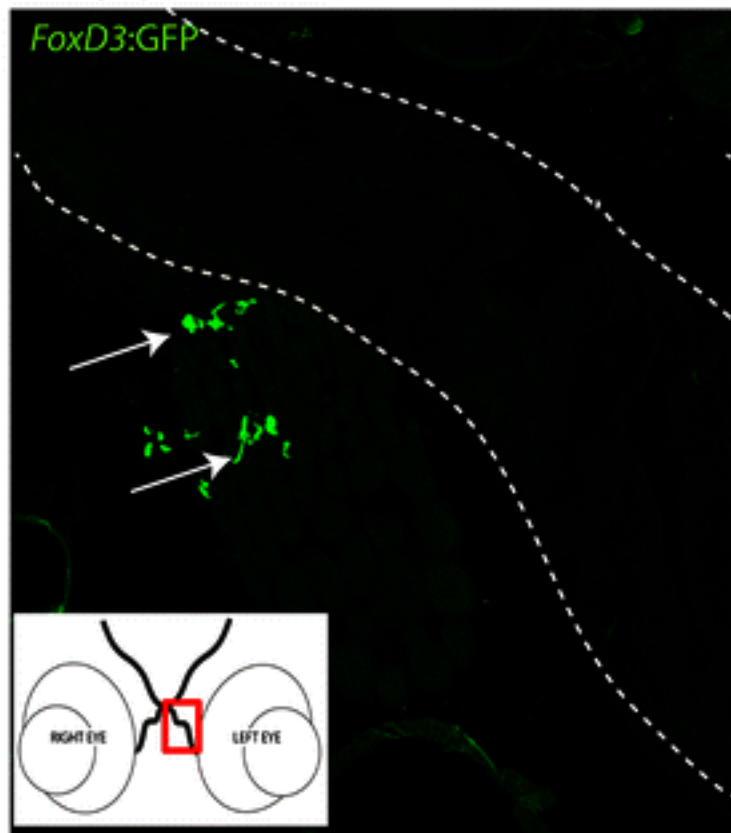
28 days post LPC lesion



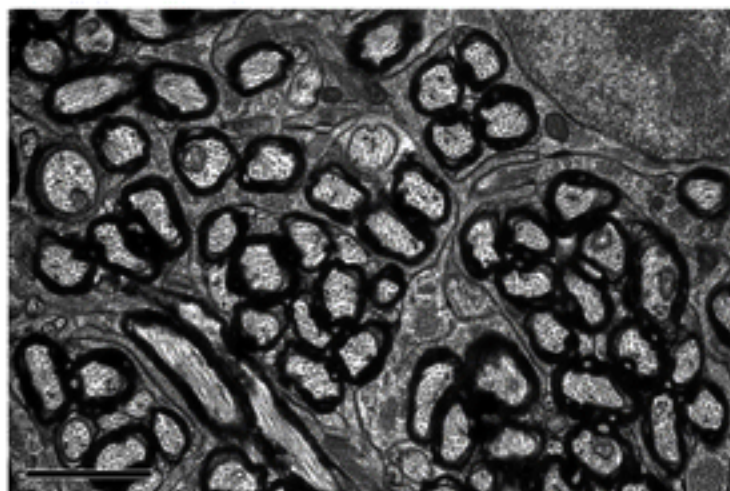
D



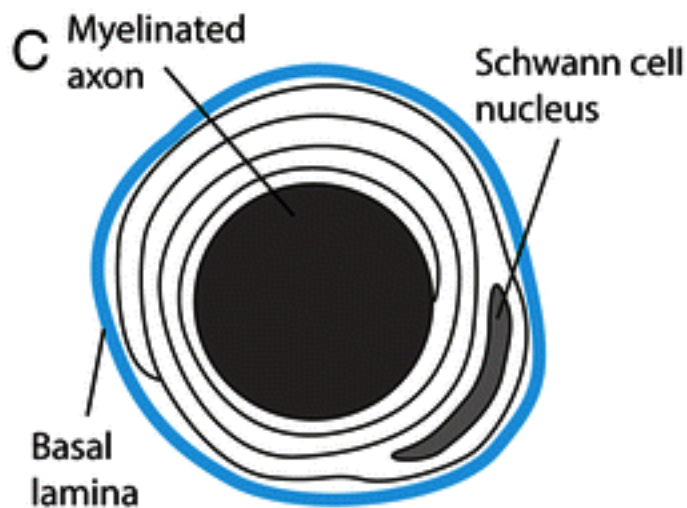
A 28 days post lesion

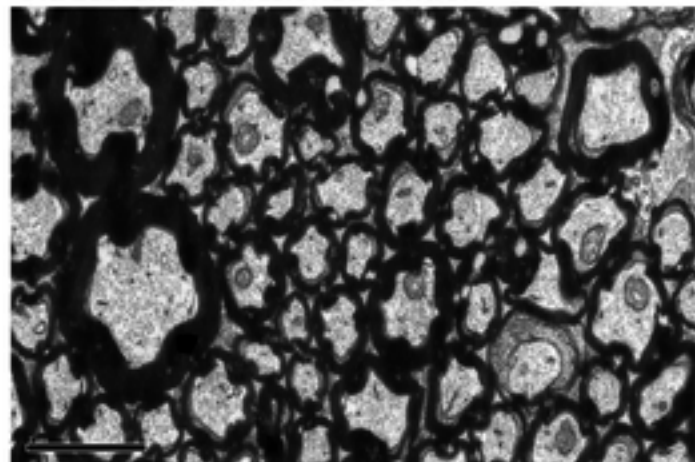


B

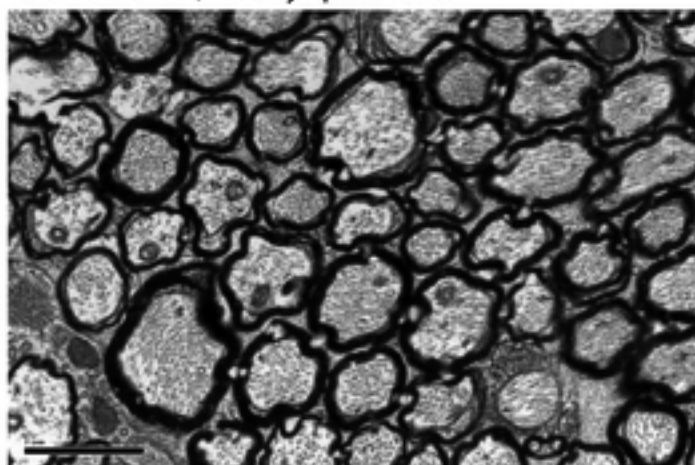


C

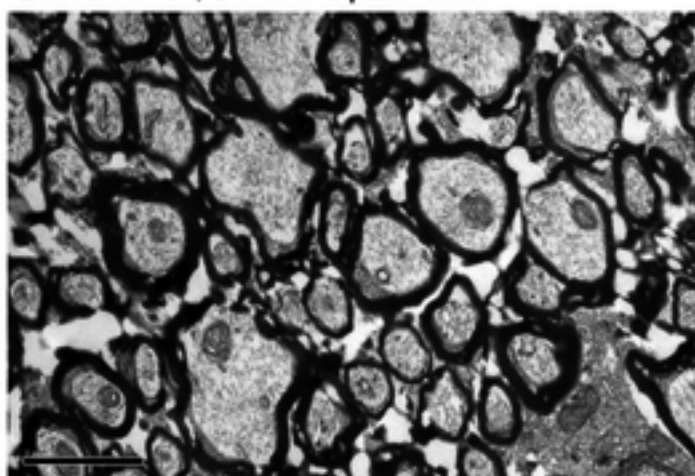


A Old zebrafish, unlesioned control

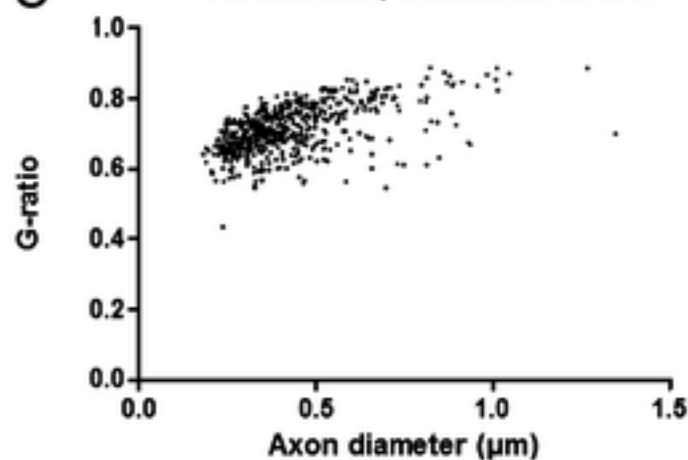
Old zebrafish, 28 days post lesion



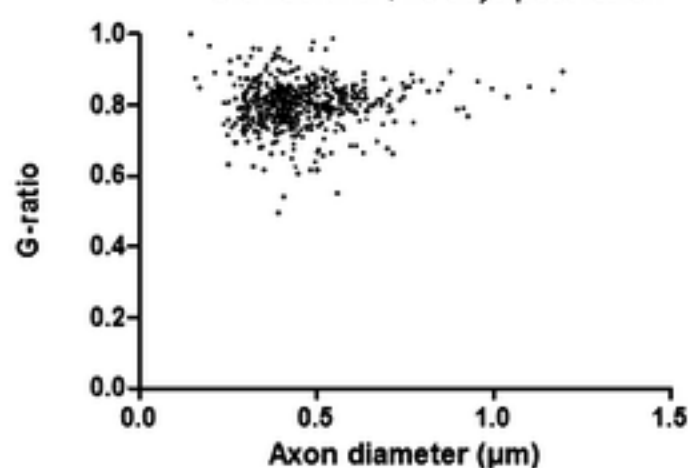
Old zebrafish, 3 months post lesion

**C**

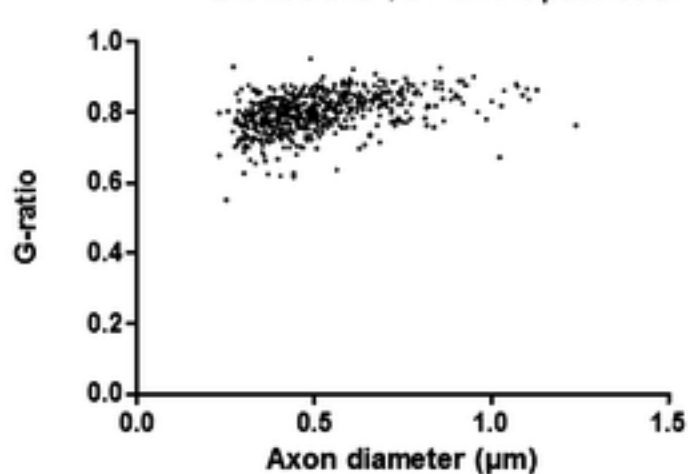
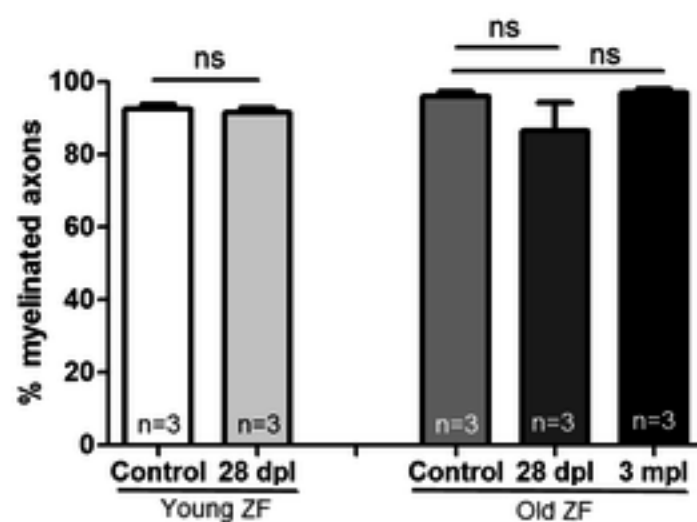
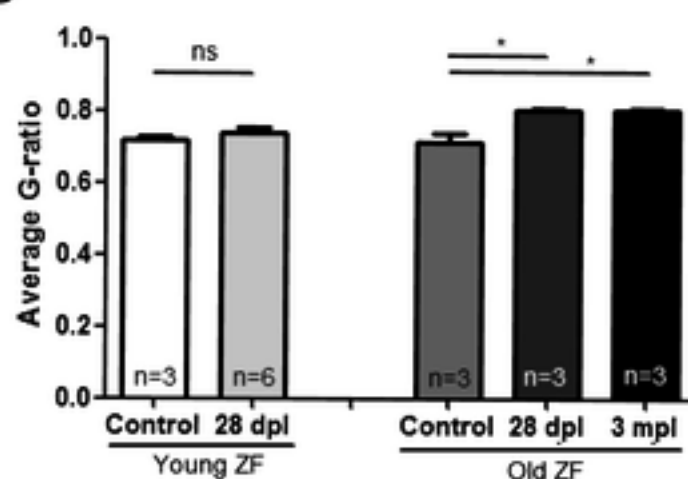
Old zebrafish, unlesioned control



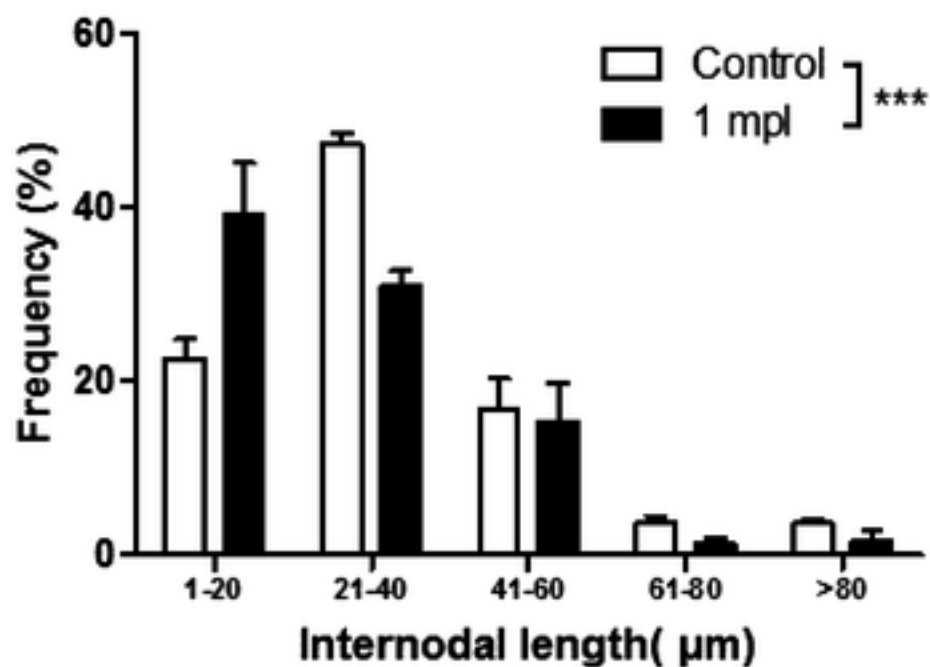
Old zebrafish, 28 days post lesion



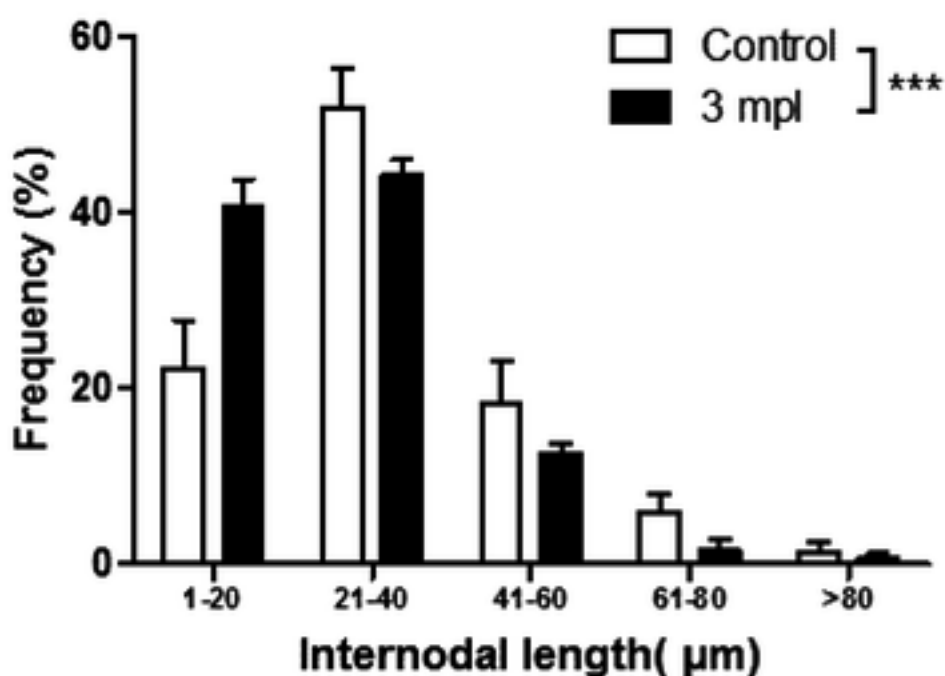
Old zebrafish, 3 months post lesion

**B****D**

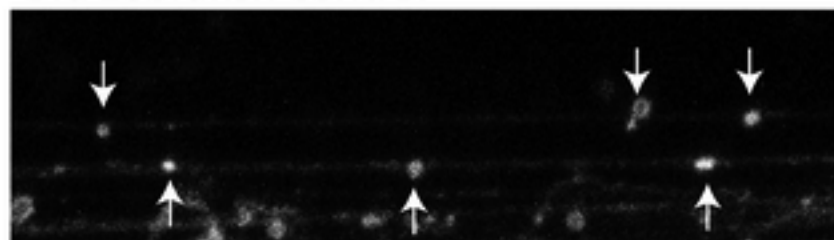
A Young zebrafish



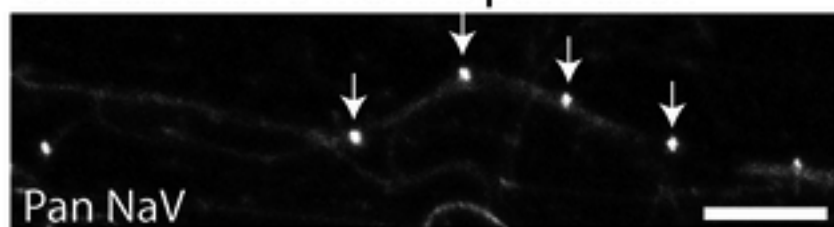
B Old zebrafish



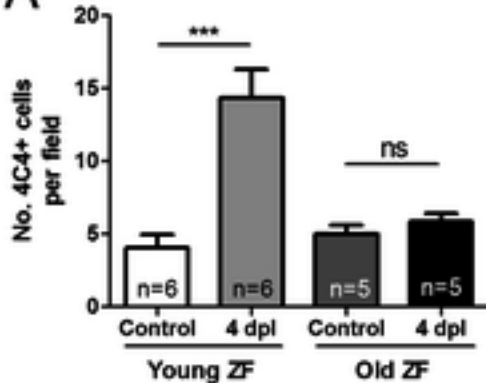
C Old zebrafish control



Old zebrafish 3 months post lesion



Pan NaV

A**B**